Chemical inhibitors of cyclin-dependent kinases

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Transient activation of cyclin-dependent kinases (CDKs) is responsible for transition through the successive phases of the cell-division cycle. Major changes in the expression and regulation of CDKs have been described in human tumours. Enzymatic screening is starting to uncover chemical inhibitors of CDKs that arrest the cell cycle at various steps. This review summarizes our knowledge of the first generation inhibitors, their molecular mechanisms of action and their effects on the cell cycle and apoptosis, and discusses their potential as synchronizing agents, as ligands for affinity chromatography and as therapeutic agents.

Cyclin-dependent kinases (CDKs) play a central role in the initiation, ordering and completion of cell-cycle events[1–3]. Human tumour development is associated with numerous alterations of CDKs and their regulators[4,5]. The importance of CDKs in cell-cycle regulation, their links with differentiation and apoptosis, their direct interactions with oncogenes and tumour suppressors, the frequent deregulation of CDKs and their regulators in cancer, and the therapeutic potential of natural inhibitors[6] have encouraged an active search for chemical inhibitors of CDKs[7]. These inhibitors block cell-cycle progression and display potentially interesting antitumour activities. CDK inhibitors are active in the submicromolar range at present, but we can anticipate the impending development of novel inhibitors acting in the subnanomolar range. This improvement will be particularly important for molecules intended for medical applications.

Screening for CDK inhibitors

Functional inhibition of CDKs can potentially be obtained through a variety of mechanisms (Fig. 1). These include competition with the substrates (ATP or protein), interaction with the T-loop phosphothreonine, interference with the binding of cyclins, with the low-molecular-weight protein subunit $p9^{CKShs}$ or with the activating cdc25 phosphatase, interference with the cellular-localization domains (cytosolic versus nuclear, microtubules), mimicking the interactions of natural protein inhibitors or interference with the cyclin-destruction signal.

Molecular interaction-based screens are beginning to reveal inhibitory (peptide) molecules[8]. However, the most frequently used screens to identify inhibitory molecules are focused on the catalytic activity of CDKs[9]. Enzymes are either purified from exceptionally rich sources (e.g. CDK1–cyclin-B from starfish oocytes)[10] or from insect cells co-infected with CDK- and cyclin-expressing baculoviruses. In the former case, the CDK1–cyclin-B kinase is purified by affinity chromatography on $p9^{CKShs}$–sepharose beads. In the latter case, an affinity moiety (encoding glutathione-S-transferase) often flanks the cyclin gene, allowing simple and efficient purification of the active CDK–cyclin complex from insect cell lysates. Kinase activities are measured, through the transfer of [^{32}P]phosphate from [γ-^{32}P]ATP to appropriate substrates (histone H1, retinoblastoma protein, synthetic peptide substrates), in the presence of increasing concentrations of the potential inhibitors. These relatively simple mechanism-based assays are particularly well suited for high-throughput screening of large collections of compounds or chemical libraries. The sources of inhibitors can be multiple – purely synthetic compounds (structure–activity studies from a lead compound), natural products (from microorganisms, plants, marine organisms, etc.) and natural extracts (for which the kinase assays serve as a purification guide for the active substance). Once an inhibitor has been identified, its molecular mechanism of action is investigated by classical enzymological analysis or co-crystallization with the kinase. It should be emphasized that purified CDKs have become a quite frequently used target for large-scale screening of potential antitumour agents by pharmaceutical companies.

CDK inhibitors – their potency and selectivity

At the time of writing this article, seven types of CDK inhibitors have been described: staurosporine[9,11] and UCN-01 (Ref. 12), two microbial alkaloids identified from *Streptomyces* sp. cultures; butyrolactone[13–15], initially identified from *Aspergillus terreus* var. *africanus*, then from *Aspergillus* strain F-25799; flavopiridol[16–19] and L868276, derived by synthesis from a parent structure extracted from the Indian plant *Dysoxylum binectariferum*; suramin[20], a naturally occurring glycosaminoglycan; 9-hydroxy-ellipticine[21], derived from a parent compound extracted from the plants *Ochrosia elliptica* and *Ochrosia acuminata*; olomoucine[22–26], roscovitine[27,28] and isopentenyladenine[9], related to 6-dimethylaminopurine (6-DMAP), itself derived from the natural product puromycin[29]; and peptides[8,30,31], some of these being derived from the sequence of the natural, protein CDK inhibitors described in the accompanying review[32]. The structures of the non-peptidic inhibitors are presented in Figure 2; their efficiency and selectivity are displayed in Table 1.

With the exception of randomly obtained peptides, all these inhibitors are derived from natural sources. This observation once more stresses the importance of microorganisms, plants and animals as the fundamental source for novel molecules as enzyme inhibitors.

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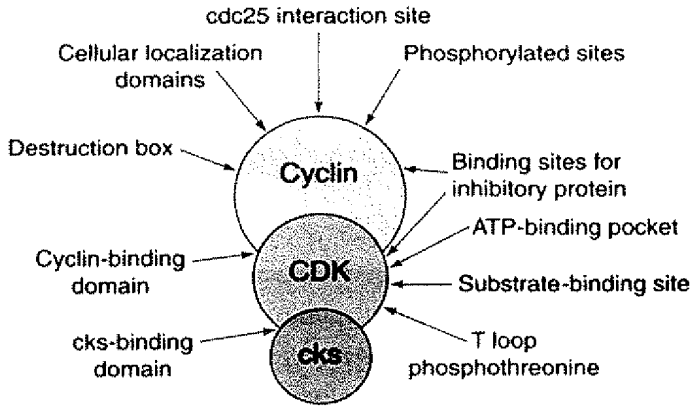

FIGURE 1

Sites for inhibition of cyclin-dependent kinases (CDKs). CDK complexes display numerous potential sites for inhibition on their catalytic subunit (CDK) or their regulatory subunits, cyclins and cks ($p9^{CKShs}$ for CDK1, CDK2 and CDK3). Interaction of a molecule with any of these sites (in a subunit-specific manner, arrows) is likely to interfere with the activity or cellular function of the CDK. The ATP-binding pocket and the substrate-binding site are presently the most heavily targeted domains.

The structures of these inhibitors differ quite widely and each represents a potential 'lead compound' from which inhibitors with greater potency and selectivity could be designed by step-by-step structure–activity studies and analysis of crystal structures of CDK–inhibitor complexes (see, for example, Refs 23 and 26–28). This variety of structures is particularly striking when considering that, for each of these chemical inhibitors to which a known mechanism of action has been ascribed (staurosporine, butyrolactone[13], flavopiridol[18,19], olomoucine[24], roscovitine[27]), the mechanism turns out to be a competitive inhibition of ATP binding. We anticipate that CDKs will be found to accommodate a large variety of inhibitory structures.

Some of the inhibitors described demonstrate remarkable selectivity. Butyrolactone-I and roscovitine even differentiate the CDK family into two subfamilies: CDK1, CDK2, CDK5 (sensitive) and CDK4, CDK6 (insensitive). The molecular reasons for the relative insensitivity of CDK4/CDK6 to several CDK1/CDK2/CDK5 inhibitors are still not understood. With the exception of the p16-derived peptides[30,31], no CDK4/CDK6-selective inhibitors have been described so far. It is hoped that new inhibitors will discriminate even further among the different CDKs despite their relatedness (CDK1 versus CDK2; CDK2–cyclin-A versus CDK2–cyclin-E, etc.). By contrast, other inhibitors show little selectivity (staurosporine, UCN-01, suramin). Although these non-specific compounds may be less interesting in cell-cycle studies, they may provide starting points for the design of selective inhibitors. This is well illustrated by the remarkable selectivity of roscovitine and olomoucine, two compounds derived from the nonselective kinase inhibitors isopentenyladenine and 6-DMAP.

Molecular interaction between inhibitors and CDKs

Understanding how inhibitors interact with CDKs progressed spectacularly as a result of the crystallization of CDK–inhibitor complexes. In addition to ATP[26], four inhibitors have been co-crystallized with CDK2: isopentenyladenine[26], olomoucine[26], roscovitine[27] and the flavone L868276 (Ref. 19; Fig. 3). All these inhibitors localize in the ATP-binding pocket, located in the cleft between the small and large lobes of the kinase[33]. The ATP-binding pocket has a surprising ability to accommodate various structures. Unexpectedly, the purine rings of olomoucine and roscovitine were found to be oriented in a totally different manner to that of the purine ring of ATP[26]. Analysis of the structures of the CDK–inhibitor complexes has allowed the identification of several amino acid residues of CDK2 that are essential for the CDK–inhibitor interaction. Ile10, Leu83 and Leu134 account for ~40% of the total contacts between CDK2 and the inhibitors. The specificity of L868276 and roscovitine/olomoucine is provided by interactions with an area that does not bind any part of ATP[19,26,27]. The interacting amino acids from this domain are not conserved in non-CDK kinases; this provides an explanation for the specificity of these compounds and may assure their importance as inhibitors of cell proliferation.

The shape complementarity between an inhibitor and its target enzyme is best described as solvent-accessible surfaces that become buried in the protein and in the inhibitor upon complex formation. If the complementarity is good, the size of both buried surfaces should be similar. The buried surfaces for roscovitine, L868276, olomoucine and isopentenyladenine amount, respectively, to 78, 75, 73 and 70% of the buried surface in CDK2. This is in agreement with the observed inhibitory efficiencies (IC50s: 0.45, 1.7, 7 and 50 μM, respectively). Obviously, the crystal structure of CDK4 will be very helpful in understanding why some molecules do not inhibit this enzyme, whereas they inhibit CDK1 and CDK2. Finally, it should be noted that only monomeric (thus inactive) CDK–inhibitor complexes have been co-crystallized. Structural data from 'active' CDK–cyclin–inhibitor complexes may reveal novel inhibitory interactions.

CDK inhibitors and cell division

Understanding what a kinase inhibitor does to a cell is not a trivial matter. The *in vivo* specificity of any CDK inhibitor always remains an open question even if strongly supported *in vitro* by proven selectivity on a battery of purified enzymes. Factors that determine the *in vivo* efficiency of the inhibitor include cell permeability, partitioning between membrane and cytosol–nucleoplasm, accumulation away from its target into a specific compartment, metabolism into inactive compounds, high concentrations of competing ATP or competing binding proteins, unidentified enzymatic targets or, most likely, a combination of these factors. For these reasons, results obtained with (CDK) inhibitors should be interpreted with caution. It is essential to demonstrate that CDK substrates are not phosphorylated *in vivo* following cell treatment with the CDK inhibitor (see examples below). The use of various chemically unrelated inhibitors (for example, flavopiridol versus olomoucine) and the use of closely related, but differentially active, compounds (olomoucine versus its inactive

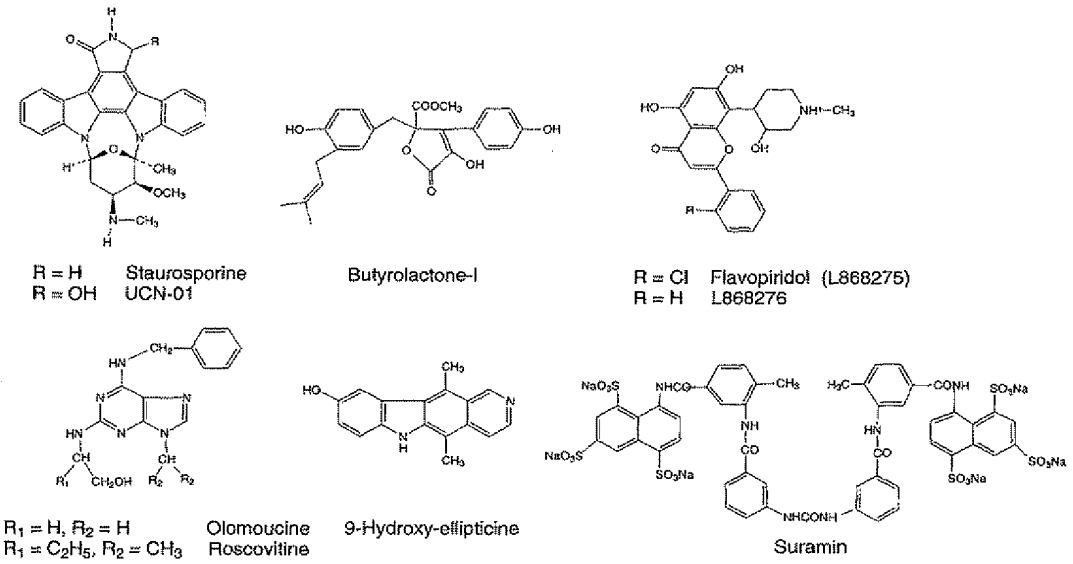

FIGURE 2

Structure of known chemical inhibitors of cyclin-dependent kinases. For selectivity and efficiency of each compound, see Table 1. Staurosporine, olomoucine and suramin are available from commercial sources.

isomer iso-olomoucine) add further support to a specific effect on CDKs. If these precautions are taken, we believe that use of chemical CDK inhibitors will elegantly complement the use of dominant–negative mutants of CDKs[34,35], the forced (over)expression of natural protein inhibitors[32], the use of the temperature-sensitive murine CDK1 mutant tsFT210 cell line[36] or microinjection of antibodies against CDKs[37].

CDK inhibitors clearly inhibit cell-cycle progression. Various CDK inhibitors have been tested on the National Cancer Institute panel of 60 human tumour cell lines[24,28]. Invariably, cell growth is inhibited. This effect is independent of the tumour-suppressor protein p53, a primary controller of the endogenous cell-cycle inhibitor $p21^{Cip1}$, as inhibition is observed whether p53 is normal or mutated/deleted (P. J. Worland and P. O'Connor, pers. commun.). As illustrated in a large variety of models with partially synchronized cells[14–16,23,24,28], chemical CDK inhibitors have two major effects on the cell cycle: arrest in late G1 phase or arrest in late prophase. These effects are difficult to detect in totally unsynchronized cells[25].

When MDA468 breast carcinoma cells are released from a nocodazole block in the presence of flavopiridol, they complete mitosis but arrest in G1 phase[16]. When they are released from an aphidicolin block in the presence of flavopiridol, the same cells arrest in late prophase[16]. Similar results have been obtained with WI38 cells treated with butyrolactone-I[13,14], or tobacco BY-2 cells treated with roscovitine

TABLE 1 – SPECIFICITY OF KNOWN CHEMICAL INHIBITORS OF CYCLIN-DEPENDENT KINASES[a]

Target	Staurosporine[7,11]	UCN-01 (Ref. 12)	Butyrolactone-I[13]	Flavopiridol[16]	Olomoucine[23]	Roscovitine[28]	9-Hydroxy-ellipticine[21]	Suramin[20]
CDK1	0.003–0.009	0.031	0.60	0.40	7	0.65[d]	~1	4
CDK2	0.007	0.030	1.50	0.40[c]	7	0.70	ND	ND
CDK4	<10 000	0.032	No effect[b]	0.40[c]	>1000	>100	ND	ND
MAPK	0.020	0.910	94	ND	30	30	ND	ND
PKA	0.008	ND	260	145	>2000	>1000	ND	656
PKG	0.009	ND	ND	6	>2000	>1000	ND	ND
PKC	0.005	0.007	160	ND	>1000	>100	ND	29–50
Tyr kinase	0.630 (EGF-R) 0.006 (Src)	ND	>590 (EGF-R)	25 (EGF-R)	440 (EGF-R)	70 (I-R)	ND	70 (EGF-R)

[a]IC50s (μM) of inhibitors tested against a series of purified protein kinases. CDK, cyclin-dependent kinase; EGF-R, epidermal growth factor receptor tyrosine kinase; I-R, insulin receptor tyrosine kinase; MAPK, mitogen-activated protein kinase; ND, not determined; PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase; Tyr, tyrosine.
[b]Y. Taya, pers. commun. [c]P. J. Worland, pers. commun. [d]IC50 for racemic mixture; IC50 for (*R*) roscovitine is 0.45 μM.

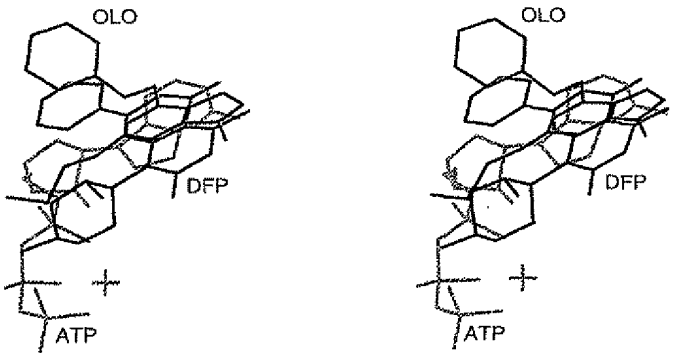

FIGURE 3

Stereo drawing of CDK2 ligands after superposition of the complex structures on Cα-atoms of CDK2. The ligands are olomoucine (OLO, green), des-chloro-flavopiridol [(L868276) (DFP, red)] and ATP (ATP with Mg^{2+} ion as a cross, orange). Figure courtesy of Sung-Hou Kim, Melvin Calvin Laboratory, University of California, Berkeley, USA.

(S. Planchais *et al.*, pers. commun.). The observed inhibition of pRb phosphorylation, a target of CDK activity, suggests that CDKs have been inhibited *in vivo*[12–14]. Cyclin A expression, a marker for late G1 phase in cell proliferation induced by Myc in fibroblasts, is inhibited by $p21^{Cip1}$, $p27^{Kip1}$ and roscovitine[38]. This G1 arrest is presumably due to inhibition of CDK2–cyclin-E and CDK2–cyclin-A.

A late-prophase arrest induced by CDK inhibitors has been described in a variety of invertebrate and vertebrate oocytes and embryos[23,24,28], as well in mammalian cell lines (Refs 16 and 28; reviewed in Ref. 7). The observation of *in vivo* inhibition of the phosphorylation of several CDK1–cyclin-B substrates strongly suggests that the target of the inhibitors is indeed CDK1–cyclin-B. For example, phosphorylation of histone H1 is inhibited *in vivo* by UCN-01 (Ref. 12), phosphorylation of the elongation factor subunits γ and δ[24,28] is inhibited *in vivo* by olomoucine and roscovitine, and phosphorylation on vimentin Ser55 [a residue specifically phosphorylated by CDK1–cyclin-B (Ref. 39)] is inhibited *in vivo* by roscovitine in L1210 cells[28]. Another CDK1-dependent event, the mitotic disassembly of the Golgi apparatus, is inhibited by olomoucine both *in vivo*[40] and *in vitro* (T. Misteli and G. Warren, pers. commun.). This block of entry into M phase is presumably accounted for by an inhibition of CDK1–cyclin-B.

CDK inhibitors and apoptosis

Multiple pathways lead to apoptosis, some of which correlate with activation of CDKs, whereas others apparently do not require them (see references in Ref. 7). Three types of effects of CDK inhibitors on apoptosis have been described. First, no effect – for example, Myc-induced apoptosis is insensitive to roscovitine, $p21^{Cip1}$ and $p27^{Kip1}$ (Ref. 38). Second, inhibition of apoptosis – in the absence of trophic factors or in the presence of a DNA-damaging agent, several neuronal cell types [such as differentiated PC12 cells[41], sympathetic neurones, cortical neurones (D. Park and E. Morris, pers. commun.)] enter apoptosis. The presence of flavopiridol or olomoucine specifically protects these neuronal cells from death. Expression of dominant–negative mutants of CDK1, CDK2 and CDK3 in HeLa cells suppresses apoptosis[35]. Furthermore, overexpression of a constitutively active CDK1, but not of wild-type CDK1, induces apoptosis in HeLa cells[42]. Forced expression of $p21^{Cip1}$ and $p16^{INK4A}$ was recently found to block apoptosis during myocyte differentiation[43]. Taken together, these results show that inappropriate activation of CDKs (resulting from an attempt to re-enter the cell cycle or CDK overexpression?) may drive some cell types into apoptosis. CDK inhibition then appears to suppress cell death. Third, induction of apoptosis – while differentiated PC12 cells are protected from cell death by olomoucine and flavopiridol, these drugs trigger apoptosis in mitotic PC12 cells[41]. Olomoucine greatly stimulates p53-independent apoptosis in cells that have been arrested in G2 by DNA-damaging agents such as mitoxantrone or *cis*-platinum[25]. The authors propose that a CDK1 is actively inhibiting apoptosis in G2 in these cells and that inhibition of this kinase results in enhanced cell death[25]. The apoptosis-inducing effects of roscovitine and olomoucine are p53-independent (P. O'Connor, pers. commun.). Taken together, these results suggest that apoptosis may be triggered in actively dividing cells by CDK inhibition.

CDK inhibitors as molecular tools

Since CDK inhibitors arrest cells both in G1 and late G2/early prophase, they can be used to synchronize cells only when combined with another synchronizing agent/method. A nice example was provided by the use of olomoucine to obtain HeLa cell populations enriched in prophase to study disassembly of the Golgi apparatus at mitosis[40]. Cells were first presynchronized with aphidicolin (accumulation in G1/S), washed, treated with olomoucine (giving a reversible prophase arrest) and then released from their arrested state by washing. Another example is provided by tobacco BY-2 cells (S. Planchais *et al.*, pers. commun.): addition of roscovitine results in a totally reversible arrest in late G1 (before histone H4 gene expression and prior to the aphidicolin-sensitive step). If cells are allowed to proceed through S phase before roscovitine addition, they arrest reversibly in prophase. The obvious requirement for the use of CDK inhibitors as synchronizing agents is complete reversibility. However, this effect has not been observed for all cell models.

We anticipate that immobilized CDK inhibitors will soon be available for affinity purification/depletion of CDKs from cellular extracts. These tools will be particularly useful for massive purification of expressed CDKs (for crystallography or screening purposes). They might also be used for comparative analysis of CDKs extracted from cells at different developmental or cell-cycle stages (variation of concentration, kinase activity, post-translational modifications, etc.). The development of this type of molecular tool is dependent upon high selectivity, efficiency, reversible binding (i.e. easy elution) and low cost.

CDK inhibitors in therapy

Through their protective effects against neuronal cell death, chemical inhibitors of CDKs may have some applications in nervous-system pathology. Owing

to their marked cell-cycle-arresting activities, CDK inhibitors are being evaluated in situations involving undesired proliferation [cancer, psoriasis, growth of fungi, parasites and plants (herbicides), etc.]. Six arguments support the efforts to evaluate chemical inhibitors of CDKs as potential antitumour agents:

- their specific cell-cycle effects (see above); highly specific compounds are more likely to have fewer secondary effects[44];
- their apoptosis-inducing effects in actively dividing cells (see above). Efficient antitumour drugs are thought to act by triggering apoptosis in the target tissue[44];
- the suppression of proliferation *in vitro* by $p21^{Cip1}$ or $p16^{INK4B}$ overexpression and *in vivo* inhibition of tumorigenicity by adenovirus-mediated $p21^{Cip1}$ gene transfer (see Ref. 32);
- the links between cyclin-D-dependent kinases, their protein inhibitors and differentiation (see Ref. 32);
- the numerous examples of abnormalities of protein CDK inhibitors in human tumours, the oncogenicity of positive regulators of CDKs (cdc25 phosphatase, cyclin E);
- the independence of the effects of CDK inhibitors towards wild-type p53 and pRb (Ref. 25, and P. O'Connor and P. J. Worland, pers. commun.).

Flavopiridol displays consistent antitumour effects *in vivo* in human tumour xenografts in athymic mice[45]. Toxicity evaluations in dogs and rats suggest that the dose-limiting toxicity is gastrointestinal (diarrhoea), with or without haematopoietic effects. Flavopiridol is the first specific CDK inhibitor to undergo clinical trials on a spectrum of malignancies. Preliminary results (E. Sausville, pers. commun.) are rather encouraging, especially because of the relative lack of toxicity of flavopiridol. The contribution of CDK inhibitory effects of 9-hydroxy-ellipticine and suramin to the demonstrated antineoplastic activity of these compounds is unknown. Clearly, both compounds act on multiple targets, and it remains to be determined whether improving their CDK inhibitory action by structure–activity studies will increase their antitumour effects.

Transforming a cell-cycle-arresting compound into a clinically useful antitumour agent is not an easy task and will require some imagination and luck! Combination with other antitumour agents/treatments is likely to be rewarding. Modulation of physiological parameters that impinge upon the CDK regulatory system must also be considered. For example, we feel that appreciation of the circadian control of the cell-division cycle will be instrumental in optimizing the use of CDK inhibitors in cancer therapy[46].

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